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Female behaviour prior to additional sperm receipt in the hermaphroditic pond snail *Lymnaea stagnalis*

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ABSTRACT

In many species, sexual resistance by females can fluctuate depending on different physiological and/or ecological conditions. Based on theory, we decided to test whether the simultaneous hermaphrodite *Lymnaea stagnalis* becomes more reluctant to mate in the female role after receipt of an ejaculate. We aimed to test whether receipt of an ejaculate affects the expression of recipient behaviours, and whether this can have a potential effect on the outcome of mating interactions. We found that the expression of Crawl-out and Biting behaviours increased significantly in recently inseminated snails. Our data also indicated that an increase in expression of these latter behaviours can have direct effects on the outcome of the mating interactions. These findings suggest that under the influence of recent insemination, this hermaphroditic snail is more reluctant to accept another mating in the female role and may try to actively discourage the mounting snail (sperm donor). Hence, despite the fact that these simultaneous hermaphrodites express both sexual functions, there can still be variation in motivation to mate in either role.

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Accessory gland products; gastropoda; multiple mating; reproductive strategies; sexual conflict

Introduction

It is widely observed in the animal kingdom that males usually prefer to mate more often than females. The difference in the contribution of additional matings to reproductive success can generally explain why males and females differ in their optimal mating rates (Bateman 1948). In contrast to male reproductive fitness of many separate sexed species, where the chances of siring a significant proportion of the offspring of females is increased with multiple mating, female fitness increases with moderate mating rates but decreases at intense mating rates (reviewed by Arnqvist and Nilsson 2000). This observed sex difference in the optimal rate of mating implies that males are expected to attempt to mate indiscriminately, while females are expected to be selective and to resist or to avoid superfluous matings (as cited in Cordero Rivera and Andrés 2002). As a consequence, sexual conflict over mating rate might be one of the most powerful forces driving the evolution of male and female reproductive strategies (as cited in Cordero Rivera and Andrés 2002; see also Parker 1979; Bradbury and Anderson 1987; West-Eberhard et al. 1987; Rice 1996; Alexander et al. 1997; Hosken et al. 2001).

Reluctance or resistance to mate by females can fluctuate depending on different physiological and/or ecological conditions (as cited in Cordero Rivera and Andrés 2002; as seen in many insect species: Arnqvist 1992; Rowe 1992; Jormalainen and Merilaita 1995; Choe and Crespi 1997; also reviewed in Gillott 2003 and Avila et al. 2011). Observations have revealed that mating can initiate behavioural and physiological responses in females after having copulated (i.e. post-copulatory). Such responses include obvious ones like inducing sperm storage and/or egg production, but also modulating processes involved in sperm competition, feeding and (re)mating behaviours (reviewed in Gillott 2003; Avila et al. 2011). When the latter example is induced by male accessory gland products, this could be seen as a female response that reflects reluctance or resistance to mate more.

At the behavioural level, either the mechanical act of mating itself or the transferred accessory gland products may induce a decrease in the willingness to remate or a change in the likelihood of remating. During this female refractory period, females do not remate for a period of time after successful mating and can actively reject (courting) males or have reduced attractiveness (reviewed in Avila et al. 2011). Examples of reduction of female attractiveness can be found in *Drosophila* and Lepidoptera. In these species, sexually receptive females produce sex pheromones to attract mates. Importantly,

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their attractiveness is reduced after mating owing to the inhibitory effect that the transferred accessory gland products (in the ejaculate) cause on the synthesis of such sex pheromones (Lepidoptera: Kingan et al. 1995). For example, as Bali et al. (1996) noted, the production of the sex pheromone declines considerably in the corn earworm moth *Helicoverpa zea* after mating and consequently affects female pheromonal calling behaviour. This effect was also observed, in the same species, when the synthesis of sex pheromone was artificially stopped after injection of combined extracts of accessory reproductive glands and ejaculatory duct (Kingan et al. 1993).

The receipt of ejaculates has not only been shown to decrease attractiveness or to induce a refractory period but also to induce aggression towards males. For instance, in *Drosophila melanogaster*, the receipt of sperm and of associated accessory gland proteins (including sex peptide) increases female aggressive behaviour towards novel courting males (Bath et al. 2017). Similar aggression is seen in species where the male genitalia cause damage to the female reproductive tract, as reported in the bean weevil *Callosobruchus maculatus* (Crudgington and Siva-Jothy 2000). To reduce the extent of this damage, females repeatedly kick males towards the end of copulation, which in the bean weevil shortens copulation and reduces damage (Crudgington and Siva-Jothy 2000). That such kicking behaviour can be mediated by accessory gland products was confirmed by injection studies that showed that such female rejection behaviour was induced (Hartmann and Loher 1996, 1999).

As illustrated by the above examples, such post-mating female behaviour has mostly been described in separate sexed species, leaving simultaneous hermaphrodites largely unexplored in this respect. The few studies on hermaphrodites that have been carried out in this direction illustrate that mating in the female function may affect female behaviour. For example, it was shown for the hermaphroditic sea slug *Siphopteron quadrispinosum*, where a traumatic injection of accessory gland substances precedes the transfer of sperm, that they generally accept mating rates above their optimal mating rate (see Lange et al. 2012). A study on the land snail *Euhadra quaesita* also reported that injury inflicted by the love dart, or accessory gland products transferred on the love dart, may be responsible for suppressing remating (Kimura et al. 2013). However, this study only looked at the end result and did not evaluate whether animals actively avoided mating attempts by potential partners. This was done in previous studies on species belonging to the freshwater snail genus *Physa* (*Physella*). These latter studies indicated that potential sperm recipients (i.e. mounted individuals) expressed shell shaking and biting to discourage and/or dislodge the sperm donor, especially when the potential sperm recipient already has sperm stored from previous matings (Wethington and Dillon 1996).

Given the previous findings that accessory gland products, that are transferred during mating, can affect the recipient in several ways (e.g. Koene et al. 2010; Nakadera et al. 2014), we decided to investigate such behavioural effects in *L. stagnalis*. We predicted that recently inseminated individuals would avoid and/or resist additional sperm receipt. To test this hypothesis, we performed an observational study in which we identified specific recipient (female) behaviours. Subsequently, we set up an experiment in which focal snails differed in their mating status in a standardized way (non-inseminated and inseminated). For this latter experiment, we observed the recipient behaviour both during non-mating interaction and when being mounted and/or courted, and we recorded the occurrence and duration of all behaviours, courtship time, time until insemination, and insemination time. Finally, in a third independent experiment, we tested whether mounted snails could potentially use these behaviours to avoid and/or reject mating partners.

Material and methods

Animals

For this study, mature specimens of *Lymnaea stagnalis* were obtained from the laboratory culture of the breeding facility of the Vrije Universiteit, Amsterdam. In this facility, the animals were housed in groups of the same age and under the following standard conditions: low-copper water of 20° ± 1° C, lettuce leaves *ad libitum* and a light:dark cycle of 12 h:12 h. Each laminar flow breeding tank had a capacity of 220 liters and a continuous water flow of 200 l/h (recirculated water with a small percentage of fresh water added continuously). All animals used in the experiment originated from the same breeding cohort, and were thus all of the same age (4 months old, mature snails) and selected to have a similar shell length 28–30 mm, with biggest within pair difference of 0.95 mm; to avoid effects of age and size (Nakadera et al. 2015).

Once animals were selected for observation and experimentation, they were each fed one 19.6 cm^2 disc of lettuce a day. This is the amount of lettuce that one snail can completely consume in a day. Prior to the experiments all individuals were treated in the same way to obtain the right conditions for the experiments. First, all experimental subjects were isolated for 7 days in 62.5 cl perforated polyethene jars, each individual placed in one jar. These jars were placed in a bigger laminar flow tank with a continuous water flow to keep the water quality constant. The isolation period was used to increase the snails' motivation to

mate in the male role (during this time, the prostate gland increases in size, providing a cue to the animal that mating in the male role is opportune; De Boer et al. 1997; Koene and Ter Maat 2007). In addition, one day prior to the experiment each individual was relocated into a fresh container, as they prefer to deposit their eggs on clean surfaces. This 'clean water stimulus' decreases the chance that snails will deposit their eggs during the experiment (Ter Maat et al. 1983), which would interfere with the observations. During the relocation, the focal snails were marked with a small dot of nail polish to be able to distinguish the snails during the experiments. The isolation period also allowed us to observe the snails' health (based on lettuce consumption) and whether they were indeed fully mature (based on the occurrence of egg laying). The few non-feeding and non-laying individuals were excluded from the experiment. For observations, similar 62.5 cl polyethene jars were used, but without perforations, and the jars were filled with 42.5 cl of tank water. The water that was used to fill the jars came from the same tank as the snails were kept in.

Female mating status and recipient mating behaviour in L. stagnalis

Before testing the hypothesis that recently inseminated snails avoid and/or resist excessive insemination, we identified all behavioural components of recipient snails. This was done during behavioural observations of 60 mature, mated snails. All behavioural components were identified by observing the mating interaction between paired snails and focusing on those acting as sperm recipients (female). After having characterized and identified all the behaviours, we set up an experiment in which we recorded recipient behaviour both during non-mating interaction and during mating interaction. This experiment contained two treatment groups, one with recently inseminated focal individuals (inseminated one day earlier; referred to as inseminated) and one with individuals that had not been inseminated for four days (referred to as non-inseminated).

In addition to scoring all these behaviours, we noted the courtship time, which is defined as the time between the start of the experimental observation and the final mounting that led to successful intromission and subsequent ejaculation, as well as the time until insemination (i.e. from the final mounting until successful intromission) and the insemination time, which is the total duration of insemination (i.e. from the start of intromission until penis withdrawal).

A total of 450 snails were isolated and 175 of these were used for the experiment outlined above (see Results: these engaged in copulations in the female role that were completed within the observation time). The 175 snails were randomly divided to create two focal treatment groups ($2 \times N = 35$, isolated for four days) and one set of sperm donors ($N = 105$, isolated for eight days). These isolation times are sufficient to, respectively, remove any physiological effect of a previous insemination (Koene et al. 2009, 2010; Hoffer et al. 2010) and increase motivation to mate in the male role (De Boer et al. 1997). For the recently inseminated treatment group ($N = 35$), on day four of isolation we allowed each snail to be inseminated once by an eight days isolated individual. All these pairs were observed to make sure that insemination took place. The control treatment consisted of individuals that were not given a mating partner on the day before the actual experiment. These are referred to as the non-inseminated treatment ($N = 35$).

On the days of observation (day five), we observed the recipient behaviour of one focal snail from each treatment group simultaneously (to avoid confounding factors such as time and day of observation); they were each size-matched with an eight-day isolated partner (i.e. individuals within each pair had the same shell size). All behavioural interactions were observed, as described above. For identification purposes, the focal individuals were marked with a small dot of nail polish on the shell. The behaviour of the focal recipients was observed continuously until the end of copulation and this time was noted as the total time of observation for these snails (the maximum time allowed for snails to complete mating at the beginning of each experiment was six hours and in the case where copulation started before the end of this time, the copulating pairs were observed until the end of copulation to be able to determine the duration of insemination). We excluded all pairs where focal individuals mated as a male first. During the observations, we made sure that the containers in which the snails were placed for observation did not vibrate or get moved because the snail in the female role responds to the slightest movement in its environment.

Female behavioural components in L. stagnalis *and outcome of mating interactions*

After having recorded the relevant behaviours, we tested whether the behaviours, displayed when being mounted and/or mated as female, have an effect on the outcome of mating interactions. For these observations, we set up pairs of snails that were isolated for one week and size-matched (and were otherwise kept as described above). In total, we observed 235 pairs of snails for a maximum of 6 h and scored all focal recipient behaviours described above until insemination was reached within the pair. Alongside the behavioural scoring, we also recorded the time they took to reach the final mounting that led up to insemination, the time until insemination (the time snails took to reach insemination) and insemination time. In addition, we recorded whether insemination actually occurred in

the observed pairs (or whether some interactions broke off before reaching that point).

Data analysis

To analyse the data, we used JMP 9 (SAS) and XLSTAT. For normally distributed continuous variables, such as courtship duration, we used a two-tailed independent sample t-test. Since some behavioural components could not be measured when no insemination occurred (see female behavioural components in *L. stagnalis* and outcome of mating interactions section), such data were excluded from analysis. Count data, such as the presence of a behavioural component and the number of occurrences, were either analysed using a Mann–Whitney U test or a Wilcoxon test.

Results

Behavioural components performed by L. stagnalis *in the recipient role and their definitions*

During the initial behavioural observations all behavioural components were identified by observing the mating interactions between all paired snails and focusing on snails acting as sperm recipients (female). These observations allowed us to characterize and identify the behaviours that individuals of *L. stagnalis* express during courtship and copulation when they mate in the female role. Some of these behaviours are expressed throughout the mating interaction (Locomotion, Crawl-out, Retraction and Floating; Figure 1), while Biting is particularly expressed when the recipient is mounted by the sperm donor and during insemination attempts (Figure 1). Obviously, a successful Swap position can only be reached once insemination has started and is included for completeness. As has been reported before (Koene and Ter Maat 2005), at the end of insemination the pair will either separate or a Role swap will take place. In the latter case, each partner now performs the opposite role, as illustrated by the line colour of the involved individuals (Figure 1).

The identified recipient behaviours (Figure 1) can be categorized as follows: 1. Locomotion is defined by the animal moving itself forward. 2. Crawl-out is defined as exiting the water, positioning more than half of the body and shell above the water line. 3. Retraction is defined as hiding in the shell. 4. Floating involves the detachment of the animal's foot from the surface and floating in the water or on the water surface. 5. Biting (generally including an apparent attempt by the mounted individual to mount the shell of the mounting animal) involves the use of the radula to bite the body or shell of the partner and an attempt to reverse the sexual role (generally when manifesting these behaviours the animal raises its own shell). 6. Role alternation position, where the inseminated individual remains positioned on the shell of the mounting animal prior and

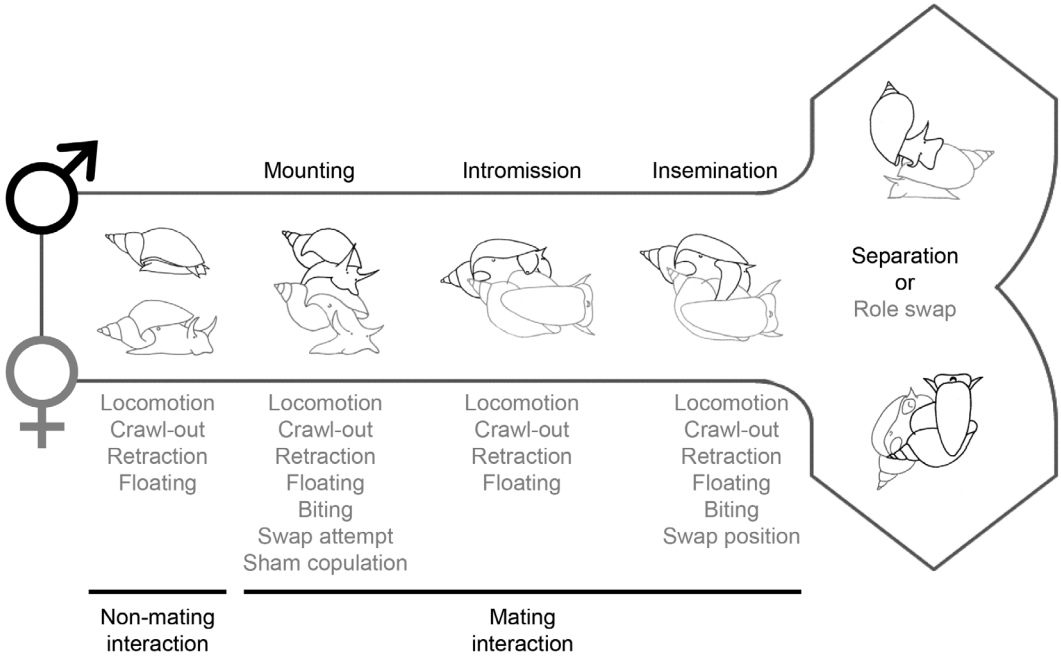

Figure 1. Overview of the different behavioural components exhibited by snails acting as female (i.e. in the sperm recipient role).
Notes: Except Biting, which is only observable during mating interactions, all the other components (i.e. Locomotion, Crawl-out, Retraction and Floating) are expressed both throughout the mating interaction and non-mating interaction. Sham copulation may be observed several times before successful intromission. The Swap position, or Role swap are seen when the female-acting snail reaches back to hold onto the shell of the mating partner, ready to mount and donate sperm itself when the first insemination has ended. The primary sperm donor is displayed in black (also indicated by the male symbol), the sperm recipient in grey (see also the female symbol); note that the roles (and colours) are swapped at the bottom right of the figure.

during successful intromission of that individual (i.e. before and/or during sperm transfer). Generally, this is the preparatory phase that precedes the exchange of sex roles just after the penis has been removed by the mounting animal (see Koene and Ter Maat 2005), but sometimes this situation may be characterized by failed attempts to attach to the shell of the mounting animal. At the end of copulation, the snails can then either separate or swap roles (most of the time preceded by the role alternation position) which is defined as the alternation of the sexual role just after the end of sperm transfer. 7. Sham-copulation, which is not necessarily related to mating behaviour of the mounted animal, can continue for 15–60 min and even longer and occurs when the preputium is placed under the shell of the female without subsequent intromission and ejaculation (i.e. missing the gonopore). It is generally characterized by strong withdrawal of the anterior part of the recipient after which it relaxes again and may resume locomotion or floating, while the partner's preputium remains in place or is withdrawn (Van Duivenboden and Ter Maat 1988).

Female mating status and recipient mating behaviour in L. stagnalis

In the 31 and 29 successful matings observed in, respectively, the non-inseminated and inseminated treatments, a total of 19 and 18 snails mated as females. Given that *L. stagnalis* also expressed some of the characterized behaviours when not mounted (i.e. outside the situation of a mating interaction), we first tested whether the identified behavioural components were expressed differently during non-mating interactions and during mating interactions for both treatment groups (i.e. non-inseminated and inseminated focals). Both the time spent on Locomotion and its frequency (i.e. locomotion events per minute) decreased significantly during mating interactions (when focal recipients were mounted) compared to the situation when snails moved around separately (non-mating interaction) in both treatments (Table 1). The same is true for the Retraction and Floating behaviours (Table 1). Biting behaviour could only be observed when the snails were in contact (i.e. during mating interaction; Table 1). The time spent on Crawl-out behaviour and its frequency (i.e. Crawl-out events per minute) increased significantly during mating interactions in the inseminated treatment, but we did not find these significant differences in the non-inseminated treatment (Table 1).

Subsequently, we considered all behavioural components (particularly those that increased in their expression when being mounted and/or courted) and tested for differences in the frequency (i.e. the occurrence of each one per unit of time) of each behavioural component. The frequency of Crawl-out differed significantly between the treatments. Inseminated snails showed Crawl-out behaviour significantly more than non-inseminated snails (Wilcoxon: $Z = -2.480$, $p = 0.013$; Figure 2(A)). We did not find differences when we compared the frequencies of the other behaviours (Wilcoxon: $Z = 0.175$, $p = 0.861$; $Z = -0.484$, $p = 0.628$; $Z = 0.680$, $p = 0.496$, for Retraction, Floating and Biting behaviours respectively; see Figure

Table 1. The proportion of total time spent (i.e. time spent on behaviour divived by total time) and the frequency (events per min) of each behaviour during non-mating interaction and during mating interaction for both the non-inseminated and inseminated treatments. The hyphen symbol (-) means that the corresponding behaviour is absent and the comparative test was not performed (in this case, Biting was not observed during non-mating interactions).

		During non-mating interaction (Mean ± SD)	During mating interaction (Mean ± SD)	Wilcoxon S	*p*-Value
Non-inseminated treatment					
Locomotion	Proportional time spent	0.778 ± 0.254	0.366 ± 0.202	−65.50	0.0008
	Frequency	0.410 ± 0.104	0.282 ± 0.098	−76.50	0.0056
Crawl-out	Proportional time spent	0.155 ± 0.210	0.250 ± 0.152	36.50	0.0887
	Frequency	0.109 ± 0.206	0.044 ± 0.030	−9.50	0.6778
Biting	Proportional time spent	-	0.076 ± 0.089	-	-
	Frequency	-	0.039 ± 0.031	-	-
Retraction	Proportional time spent	0.011 ± 0.030	0.132 ± 0.138	52.50	0.0001
	Frequency	0.004 ± 0.012	0.027 ± 0.024	41.50	0.0017
Floating	Proportional time spent	0.008 ± 0.023	0.092 ± 0.206	43.50	0.0040
	Frequency	0.009 ± 0.021	0.036 ± 0.034	39.50	0.0034
Inseminated treatment					
Locomotion	Proportional time spent	0.876 ± 0.182	0.423 ± 0.242	−75.50	<0.0001
	Frequency	0.352 ± 0.111	0.217 ± 0.074	−76.50	<0.0001
Crawl-out	Proportional time spent	0.110 ± 0.184	0.294 ± 0.215	49.00	0.0092
	Frequency	0.041 ± 0.048	0.075 ± 0.039	59.00	0.0010
Biting	Proportional time spent	-	0.028 ± 0.030	-	-
	Frequency	-	0.033 ± 0.032	-	-
Retraction	Proportional time spent	0.010 ± 0.028	0.103 ± 0.097	27.50	0.0020
	Frequency	0.010 ± 0.027	0.029 ± 0.032	25.50	0.0059
Floating	Proportional time spent	0.009 ± 0.018	0.117 ± 0.212	45.50	0.0023
	Frequency	0.007 ± 0.015	0.039 ± 0.031	50.50	0.0004

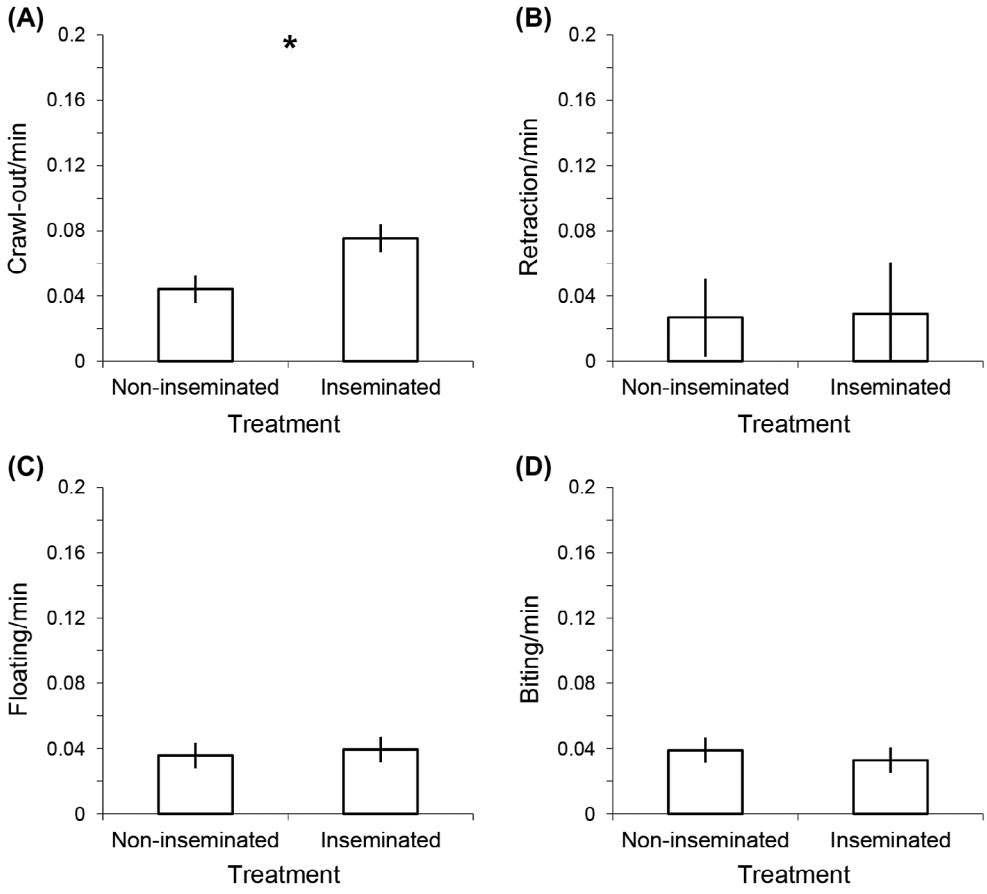

Figure 2. The frequencies (events per min ± SE) of Crawl-out behaviour (A), Retraction behaviour (B), Floating behaviour (C) and Biting behaviour (D) compared for the two treatments, non-inseminated and inseminated.

2(B), 2(C), 2(D)). To confirm that differences in recipient behaviour are related to being courted (i.e. mounted), we also analysed their frequencies from the point of mounting (i.e. the occurrence of each behaviour per unit of time during mouting only). This analysis revealed that Crawl-out and Biting behaviours increased significantly when snails were mounted in the inseminated treatment compared to the non-inseminated treatment (Wilcoxon: Crawl-out, $Z = -2.140$, $p = 0.033$, and Biting, $Z = -2.342$, $p = 0.019$, but we did not find a significant difference for Retraction and Floating, Wilcoxon: $Z = 1.673$, $p = 0.094$, $Z = 1.008$, $p = 0.313$, respectively, see supplementary Figure S1). No significant differences were found in the time it took pairs of snails to reach insemination and insemination duration ($t_{31} = -1.235$, $p = 0.887$; $t_{31} = 0.495$, $p = 0.312$, respectively). However, the time to the final mounting (leading to insemination, i.e. courtship) was significantly higher in the inseminated treatment ($t_{31} = -3.0466$, $p = 0.0047$, Figure 3).

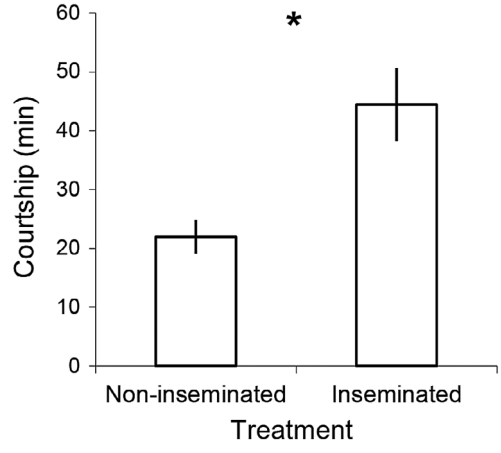

Figure 3. The time (± SE) it took pairs to reach the final mounting that led to insemination (i.e. courtship) for the two different treatments.

Female behavioural components in L. stagnalis *and outcome of mating interactions*

We tested whether the occurrence of the identified behavioural components affected the duration and outcome of mating. In the pairs, where Crawl-out was recorded it took significantly longer for the partner to reach insemination ($t_{20} = 2.530$, $p = 0.020$; Figure 4(A)) but insemination time seemed unaffected ($t_{20} = -0.570$, $p = 0.575$; Figure 4(B)).

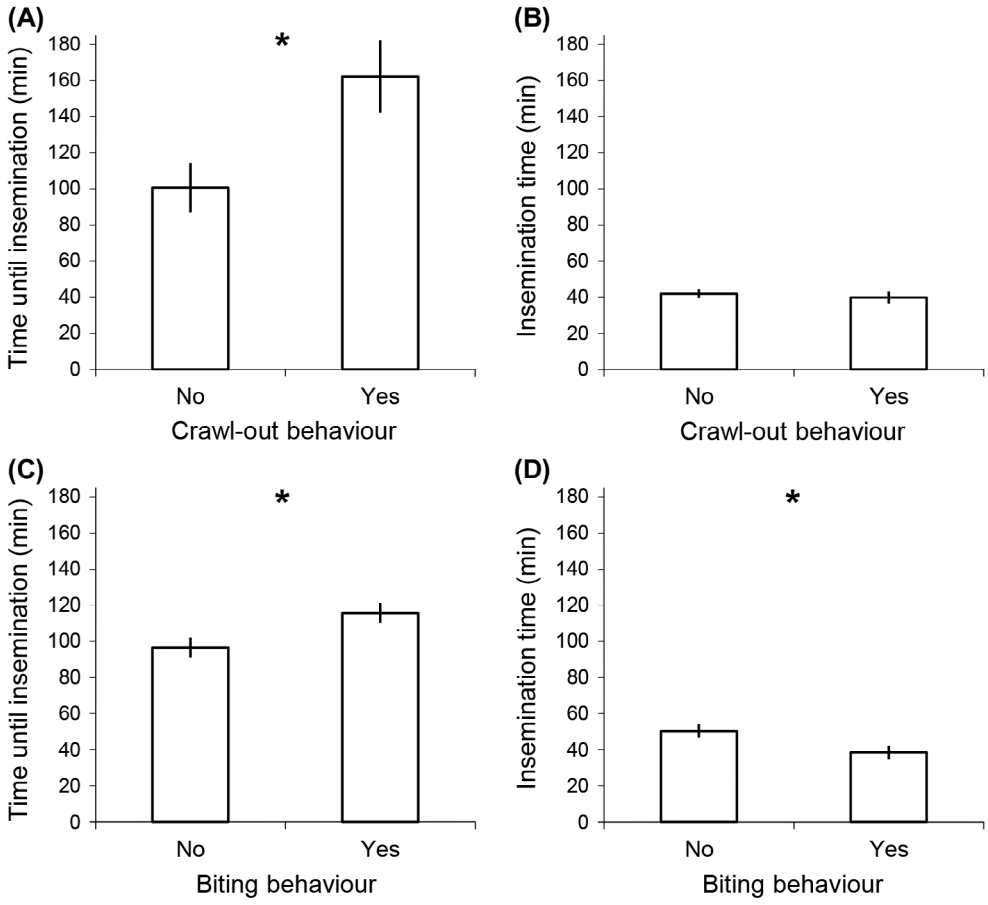

Figure 4. (A) Comparison of the average time (± SE) until pairs reached insemination for mating interactions in which Crawl-out behaviour was observed (Yes) or not (No). (C) Comparison of the average time (± SE) until pairs reached insemination for mating interactions in which Biting behaviour was observed (Yes) or not (No). (B) Comparison of the insemination time for mating interactions in which Crawl-out behaviour was observed (Yes) or not (No). (D) Comparison of the insemination time for mating interactions in which Biting behaviour was observed (Yes) or not (No).

Likewise, when focussing on Biting, the time interval to reach insemination was also significantly longer than when no Biting occurred within a mating pair ($t_{56} = 2.443$, $p = 0.018$; Figure 4(C)) and insemination time was significantly shorter ($t_{56} = -2.262$, $p = 0.028$; Figure 4(D)). The other behavioural components (Locomotion, Retraction and Floating) did not result in an increase in this time interval. By the end of the observation period, most pairs had reached insemination. As a result, we did not find a difference in the number of successful inseminations between pairs showing particular behavioural components (total number of successful inseminations 58; Mann–Whitney U test: $p > 0.05$).

Discussion

After identifying all behaviours performed by the sperm recipient of *L. stagnalis*, we specifically assessed the female behaviour and outcome of mating interactions prior to additional sperm receipt. We did so by testing whether recently inseminated snails can avoid additional inseminations when being mounted and courted by additional sperm donors. Our results revealed that the Crawl-out response (i.e. Crawl-out behaviour) increased significantly when the recipient had previously been inseminated. This behaviour is known to be performed by *L. stagnalis* in response to the potential risk of predation (Snyder 1967; Rundle and Bronmark 2001), and is even expressed when only the odour of the predator is present (Dalesman et al. 2006). Similar behaviour was also observed in the freshwater snail species *Physella gyrina* and *P. integra* in response to predation risk: they move to the surface and avoid covered habitats (Crawl-out) or they seek out covered areas (Turner et al. 1999; Bernot and Turner 2001). According to the prediction in the present study that *L. stagnalis* becomes more reluctant to mate as recipient after recent receipt of an ejaculate, the observed increase in expression of Crawl-out behaviour in recently inseminated snails (also confirmed at the point of mounting, see supplementary Figure S1), combined with increase in courtship time, support the

assumption that Crawl-out behaviour can be seen as an avoidance response that is displayed in two different situations with two different purposes (i.e. co-option of the behaviour for anti-predator and anti-insemination).

Besides Crawl-out behaviour, our results showed that the expression of most indentified behaviours (Biting, Retraction and Floating) increased significantly in the two treatments once the snails were mounted compared to when they were moving around separately. The only scored recipient behaviour that was found to decrease when mounting took place was Locomotion. Biting behaviour, which has been suggested to be used in contest escalation in *Physa* to help shake off the courting snail (DeWitt 1991; McCarthy and Sih 2008), seems also to be used by *L. stagnalis*, especially at the point of mounting.

The data of our second experiment (i.e. female behavioural components in *L. stagnalis* and outcome of mating interactions) indicate that the increased expression of some of these recipient behaviours affects the outcome of the mating interactions. Both Crawl-out and Biting were found to increase the time it took until successful intromission, although all pairs eventually reached insemination. In our study, insemination avoidance was eventually not successful, which could be owing to the restricted space in which snails were tested. However, insemination duration did increase, which indicates that some discouragement of the sperm donor may have been achieved. These findings indicate that Biting and Crawl-out behaviours can be interpreted as being part of an evasive strategy of the mounted snail in *L. stagnalis*. This is in agreement with earlier work on different freshwater species. For example, Wethington and Dillon (1996) found that previously mated snails of *P. gyrina* were able to reject unwanted mating partners more often, using similar recipient behaviours and shell shaking (that they also display for predator avoidance). Unlike *Physa*, *Lymnaea* was not found to use shell shaking, which may have to do with the fact that the latter species is much larger and that using this as a strategy to dislodge the partner from the shell may not work for large body sizes.

As reported by Wethington and Dillon (1996), in previous work, it has been assumed that hermaphroditic freshwater snails are always receptive in the female role (Van Duivenboden and Ter Maat 1985), but other studies have shown that rejection behaviour may exist in these freshwater snails (Barraud 1957; Rudolph 1979; Van Duivenboden and Ter Maat 1988; DeWitt 1991). Our results clearly agree with the latter results and indicate that these hermaphrodites may also be in conflict about when to mate, as is widespread throughout the animal kingdom (Parker 1979). As our data show, when *L. stagnalis* has been recently inseminated, it does seem more reluctant to accept another mating in the female role and may try to actively discourage the mounting snail (sperm donor). Given that the physiological effects caused by accessory gland proteins, that are present in the ejaculate, seem not all beneficial for the recipient (e.g. Sprenger et al. 2008; Koene et al. 2010), this may explain why additional inseminations are discouraged. It could also be that this behaviour is induced by accessory gland proteins, in the benefit of the first sperm donor, but this remains to be tested for this snail species.

These interpretations of our findings are in line with work on many separate sex species where remating avoidance is influenced by received seminal fluid products (reviewed in Gillott 2003 and Avila et al. 2011). Such behavioural post-mating changes are initiated in females of many insect species after the transfer of spermatophores, spermatozoa and male accessory gland secretions (reviewed in Gillott 2003 and Avila et al. 2011). These modifications of mating behaviour include decreasing receptivity (i.e. reducing female likelihood of remating) by formation of a physical barrier to reinsemination (i.e. mating plug), induction of female refractoriness (i.e. females actively reject males), and reduction of attractiveness (Avila et al. 2011). For example, in the study of Hihara (1981), the seminal fluid was directly involved in the induction of such behaviours in mated females. That study found that males that continue to transfer sperm but transfer decreasing amounts of accessory gland fluid (repeatedly mated males) become progressively less effective at inducing long-term refractoriness (Gillott 2003). Other examples of male-induced post-mating refractoriness or mating refusal come from *Aedes aegypti* where females reject mating attempts by additional partners (Fuchs et al. 1969; Fuchs and Hiss 1970; Lee and Klowden 1999), and *D. melanogaster* where females actively reject courting males after receipt of seminal fluid (Chapman et al. 2003; Liu and Kubli 2003; Yapici et al. 2008; Hasemeyer et al. 2009; Ram and Wolfner 2009; Yang et al. 2009). In general, such decreased likelihood of receiving additional inseminations indirectly affects the intensity of sperm competition, and is thus beneficial for the sperm donor. As our study now shows, this also has implications at the behavioural level of the simultaneous hermaphrodite studied here, indicating that despite the fact that they express both sexual functions, there can still be variation in motivation to mate in either role.

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